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ISOLATION AND TOXICITY OF *CYLINDROSPERMOPSIS RACIBORSKII* FROM AN ORNAMENTAL LAKE

PETER R. HAWKINS,[1] NIMAL R. CHANDRASENA,[1]
GARY J. JONES,[2] ANDREW R. HUMPAGE[3] and IAN R. FALCONER[3]
[1]AWT EnSight, 51 Hermitage Road, West Ryde, NSW 2114, Australia; [2]CSIRO Division of Water Resources, PMB 3, Griffith, NSW 2068, Australia; and [3]Department of Clinical and Experimental Pharmacology, University of Adelaide, Adelaide, South Australia 5005, Austrialia

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P. R. Hawkins, N. R. Chandrasena, G. J. Jones, A. R. Humpage and I. R. Falconer. Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. *Toxicon* **35**, 341–346, 1997.—In Australia, the tropical/subtropical cyanobacterium *Cylindrospermopsis raciborskii* forms substantial blooms in some drinking water supply reservoirs, rivers and recreational water bodies during the warmer months of the year. This paper describes the isolation, culture and toxicity characterisation of *Cylindrospermopsis* from a water bloom in a small lake in NSW, Australia. The cyanobacterium grew as straight trichomes terminating with a characteristic heterocyst. The toxic alkaloid cylindrospermopsin was separated and identified by high-performance liquid chromatography at a concentration of 5.5 mg/g dry cells, 0.026 pg/cell. Intraperitoneal injection of sonicated cells caused liver, kidney, intestinal and lung damage, with an LD_{50} of 52 mg cells/kg mouse body weight at 24 hr, and 32 mg/kg at 7 days. The 24 hr mouse toxicity is not consistent with previous studies using pure cylindrospermopsin, and is suggestive of other toxic compounds in this isolate. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The increasing identification of toxic cyanobacteria in drinking water supply reservoirs has alerted health authorities to the need for water monitoring and toxicity measurement of potentially harmful organisms (Ressom *et al.*, 1994). Most research efforts have been focused on the cyanobacterial bloom genera *Microcystis*, *Anabaena*, *Nodularia*, *Aphanizomenon*, *Nostoc* and *Oscillatoria* (Carmichael and Falconer, 1993). The former three genera commonly form blooms in temperate Australian waters, whereas the latter three do not (Jones, 1994). Furthermore, in tropical or subtropical regions, which comprise more than a third of Australian surface waters, the nitrogen-fixing cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju may be the dominant bloom-forming species. The most harmful bloom of *C. raciborskii* occurred in Solomon Dam, Palm Island, North Queensland, in 1979, causing severe hepatoenteritis. The majority of cases were in children, 138 being reported. The liver was enlarged in all cases and the initial symptoms resembled hepatitis with abdominal pain. Kidney malfunction and profuse bloody diarrhoea followed (Byth, 1980). The symptoms followed the application of copper sulfate to a dense algal bloom in the water supply dam. This outbreak was further clarified in subsequent papers by Bourke *et al.* (1983) and Hawkins *et al.* (1985) in which the causative organism and the nature of toxicity were identified. Another bloom of *C. raciborskii* in an Australian drinking water supply occurred in the North Pine Reservoir near Brisbane, Queensland. Water from this reservoir, as a consequence, was unavailable for supply to the city of Brisbane between March and June 1995. A review of the epidemiology of gastrointestinal illness and known infectious diseases from early colonial Australia (Hayman, 1992) suggested that cylindrospermopsin poisoning was possibly responsible for the debilitating and sometimes fatal disease, colloquially known as the 'Barcoo spews', once widely known in northern and central outback Australia.

The health risk posed by occurrence of *C. raciborskii* in public water supplies has made more urgent the need to dispel confusion over the identification of this species, which has arisen because the genus has been previously ascribed to *Anabaenopsis*, *Raphidiopsis* and *Cylindrospermum*. The taxonomic position of *Cylindrospermopsis* is close to *Cylindrospermum* but these two genera can be distinguished by the presence of gas vacuoles, the shape of the terminal heterocyst and the true planktonic nature of *Cylindrospermopsis* (Jeeji-Bai *et al.*, 1977). *Cylindrospermopsis raciborskii* is highly variable in trichome width and the length of the heterocysts but, according to Jeeji-Bai *et al.* (1977), the trichomes are always straight. Specimens with coiled trichomes are proposed to belong to the species *Cylindrospermopsis philippinensis*.

Cylindrospermopsis raciborskii is a cosmopolitan cyanobacterium which has an optimum temperature for growth above 25°C (Vinogradska, 1974; Hawkins, unpublished observations). Although this species is most often recorded in the tropics and subtropics, its distribution can extend into warmer regions of temperate Australia (Baker, 1991).

The original culture isolate from Palm Island, Queensland, had a 24 hr LD_{50} of 64 mg/kg (Hawkins *et al.*, 1985). This toxicity is similar to reports for other highly hepatotoxic or neurotoxic cyanobacteria, and reflects the similarities in toxic potencies between cylindrospermopsin, microcystins, anatoxins and paralytic shellfish poisons (PSPs) (all within a range of less than 10-fold), and similarity in toxin content of highly toxigenic strains. Other *C. raciborskii* bloom samples collected in Australia have shown variable toxicity in mouse bioassays (Baker and Humpage, 1994), including a 1992 sample from Solomon Dam, Palm Island, collected 7 years after the original poisoning (Queensland Water Quality Task Force, 1992). Non-toxic samples have also been collected from Australian rivers and water supply reservoirs.

To date, only one isomer of cylindrospermopsin has been isolated, and this was the case with the strain examined here. However, given the chemical plasticity that has been observed with microcystins (more than 50 related compounds), anatoxins (methylated and acetylated variants), and PSPs (non-sulfated, monosulfated and disulfated and decarbamoylated variants) it would be surprising if closely related compounds were not isolated in the future. The chemical structure of this novel alkaloid toxin (cylindrospermopsin) was elucidated in 1992 (Ohtani *et al.*, 1992) and chemical screening methods for the identification and quantification of the compound have been recently developed (Harada *et al.*, 1994). Cylindrospermopsin has now been identified in another cyanobacterium, *Umezakia natans* (Terao *et al.*, 1994).

In this paper, we report for the first time quantitative cylindrospermopsin content determined by high-performance liquid chromatography (HPLC) and concurrent mouse toxicity measurements of a toxic isolate of *C. raciborskii*, and provide details of successful strain isolation and culturing. An apparent discrepancy between the published toxicity of pure cylindrospermopsin and the toxicity of this Australian isolate of *C. raciborskii* is also highlighted.

METHODS, RESULTS AND DISCUSSION

Cylindrospermopsis raciborskii was identified in mid-summer as the dominant species in a dense bloom sample from an ornamental lake (Oatley Pond) in Sydney, Australia (33°50′S 151°04′E). Sydney is in the warm temperate zone of Australia, with summer air temperatures frequently reaching the mid–high thirties. The buoyant cyanobacterial cells were first concentrated by standing overnight. The buoyant *C. raciborskii* filaments were harvested from the surface in several small aliquots. This concentrate was used to inoculate a liquid growth medium (Jaworski's medium; Thompson *et al.*, 1988) which was modified by excluding all nitrogen sources. After 14 days, successful cultures in the liquid medium were streaked on to agar plates (0.5% agar). Single trichomes were then transferred from these plates back into Jaworski's medium for maintenance. One strain was isolated and used for mass culture, incubated at 28°C (Strain AWT 205). The mass cultures were grown in 1 litre of medium in 3 litre Erlenmeyer flasks with illumination by natural or artificial light (cool white fluorescent) at an intensity of 100–200 μEm^2/sec. The isolate (AWT 205) grew without aggregation or sedimentation in batch culture, so no agitation was necessary. Cultures were harvested in late exponential or stationary phase at cell densities of greater than 1×10^6 cells/ml, by centrifugation, and freeze-dried.

The cell count for freeze-dried *C. raciborskii* filaments was $2.10 \pm 0.3 \times 10^8$ cells/mg dry weight.

This strain has now been maintained in low growth conditions at the ambient laboratory temperature of 23°C with retention of the straight trichome habit for more than 12 months.

Cylindrospermopsin was extracted from freeze-dried cell samples in 5% aqueous acetic acid (10 mg cells/ml) by repeated ultrasonication (6 × 20 sec, 50 W). The extracts were clarified by centrifugation (4000 *g*, 10 min) and analysed by reverse-phase HPLC without further purification. The analytical method followed was a modification of that described by Harada *et al.* (1994), with a 10 min linear gradient of 0–5% methanol followed by a 10 min isocratic period of 5% methanol. This mobile phase protocol was found to overcome retention problems highlighted by Harada *et al.* (1994), using isocratic 5% methanol without a gradient. The column used was supplied by Alltech, with a packing of Spherisorb ODS-2, 5 μm, and detection was at 260 nm. The putative cylindrospermopsin peak was confirmed by co-injection with a standard (kindly supplied by Prof. Ken-Ichi Harada) and by comparison of the UV spectrum obtained with a Spectra-Physics Rapid Spectral Scanning detector, with those previously published (Harada *et al.*, 1994). As pure (solid) cylindrospermopsin is not commercially available for use as a primary standard, cylindrospermopsin was quantified using a secondary standard supplied by Prof. Harada. The concentration of the secondary standard was confirmed spectrophotometrically using a molar absorptivity of 6100 at 262 nm (Harada *et al.*, 1994).

Although several peaks were observed in HPLC chromatograms of *C. raciborskii* culture extracts (Fig. 1), only one peak had a UV spectrum identical to cylindrospermopsin [Fig. 2(a)]. The pure toxin is characterised by a distinct absorbance maximum of 262 nm,

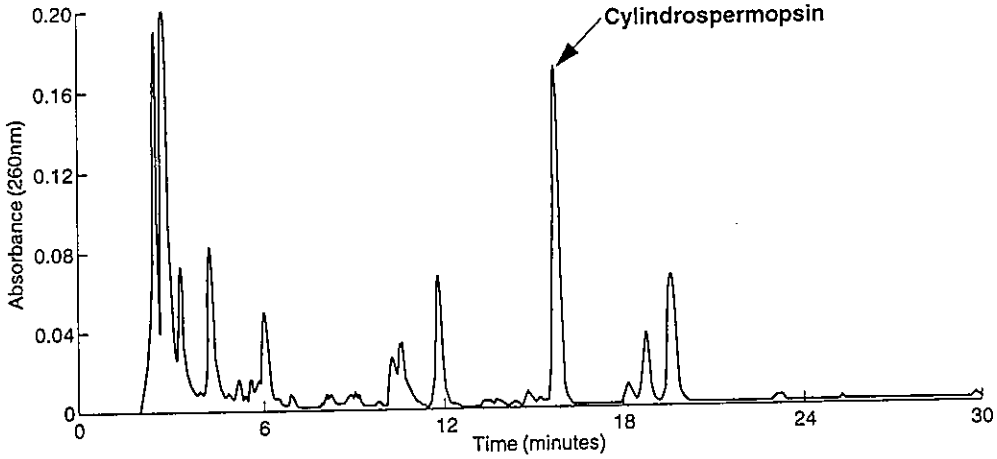

Fig. 1. HPLC chromatogram (260 nm) of the separation of a crude aqueous extract of *C. raciborskii* strain AWT205.
Cylindrospermopsin peak is identified, other peaks did not have appropriate UV spectra.

due to the uracil moiety [Fig. 2(b)] (Ohtani *et al.*, 1992). This peak also eluted with pure cylindrospermopsin when co-injected. The cylindrospermopsin content of the freeze-dried cells was determined to be 5.5×10^{-3} g/g dry weight cyanobacteria, which corresponds to 0.026 pg/cell. This cylindrospermopsin content determined by HPLC is consistent with the gravimetric measurements of Ohtani *et al.* (1992). These authors reported purification of cylindrospermopsin from the Solomon Dam, Palm Island, isolate of *C. raciborskii* at 0.5% yield (m/m).

Toxicity testing was carried out by a single i.p. injection into 52 Swiss Albino male mice (20–30 g body weight) of a sonicated (2 min) suspension of a freeze-dried culture sample

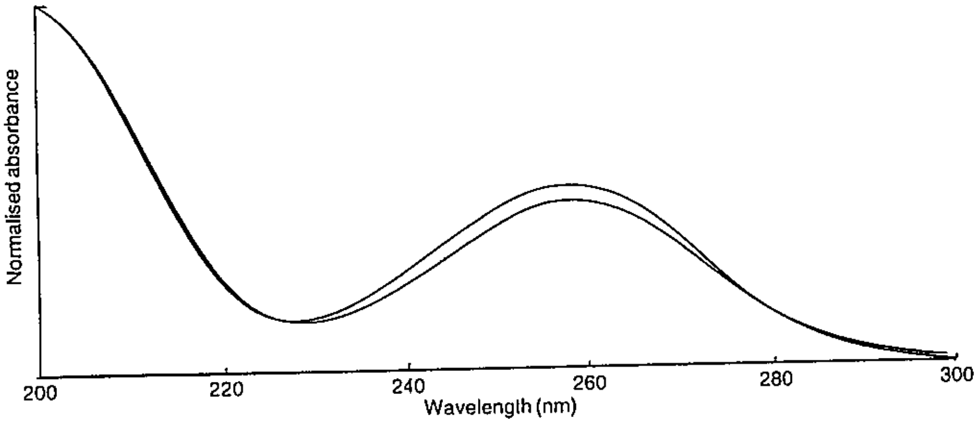

Fig. 2. UV spectra (200–300 nm) of (a) putative cylindrospermopsin peak labelled in Fig. 1 (upper spectrum), and (b) pure cylindrospermopsin (lower spectrum).

in 0.9% (w/v) NaCl solution. The dose administered ranged from 10 to 300 mg dry weight cyanobacteria/kg mouse live weight. Mice were killed by cervical dislocation when their clinical condition was assessed to be terminal, the time of death was recorded, and the mice were autopsied. The time of death ranged from 7.5 to 151 hr. All remaining animals were killed and autopsied at 168 hr. Post mortem examination showed signs of haemorrhage within the liver, kidneys and small intestine, and congestion of the lungs, at high *C. raciborskii* doses. Histopathology of the liver demonstrated centrilobular necrosis at lower doses and generalised necrosis at higher doses. Kidney damage appeared to be restricted to the tubular epithelium. The 24 hr LD$_{50}$ was calculated to be 52 mg/kg (95% confidence limits 30–77 mg/kg) and over 7 days (168 hr) to be 32 mg/kg (95% confidence limits 1–48 mg/kg).

Calculation of the theoretical LD$_{50}$ for the freeze-dried cells at 24 hr and 5–6 days, using the toxicity data published by Ohtani *et al.* (1992) for pure cylindrospermopsin of 2.1 mg/kg body weight at 24 hr and 0.2 mg/kg at 5–6 days, resulted in predicted cell toxicities of 382 mg/kg at 24 hr and 36 mg/kg at 5–6 days. By contrast, the experimentally determined toxicities were 52 mg/kg at 24 hr and 32 mg/kg at 7 days. Thus there is close agreement over the 5–7 day toxicity, but a large difference between predicted and actual 24 hr toxicity.

The original Palm Island strain used for extraction by Ohtani *et al.* (1992) had earlier shown a 24 hr toxicity of 64 mg cells/kg body weight of mice (Hawkins *et al.*, 1985), close to the value obtained in this study. Assuming the accuracy of the Ohtani *et al.* (1992) data, the discrepancy between cell toxicity and pure cylindrospermopsin toxicity at 24 hr can be hypothesised to be due to (1) the presence of cell toxins other than cylindrospermopsin, which are more rapidly lethal; (2) mouse sensitivity differences; or (3) synergistic effects from other cellular components on toxicity at 24 hr.

Since each of the toxin 'families' so far identified from cyanobacteria contains structural variants with differing toxicities, the first hypothesis appears most likely.

The biochemical mechanism of *Cylindrospermopsis* toxicity appears to be related to the inhibition of protein synthesis, and consequent metabolic changes including hepatocyte death and lipid accumulation in the liver (Terao *et al.*, 1994). It is not known whether the original alkaloid is the toxic agent or a metabolite. Evidence of glutathione depletion in hepatocytes, a role for cytochrome P450, and centrilobular cell injury, point towards a toxic metabolite (Runnegar *et al.*, 1994). However, the evidence of kidney, intestine and lung damage shows non-selectivity of toxic attack in tissues less likely to metabolise the alkaloid.

The *C. raciborskii* isolate described in this paper was comparable in toxicity with the strain isolated from the Palm Island water supply reservoir, the source of the 'Mystery Disease' in 1979, and contained 5.5 mg cylindrospermopsin/g dry weight cyanobacteria or 0.026 pg/cell. It is of interest to note that a highly toxic *Microcystis aeruginosa* sample contained 0.2 pg microsystin/cell (Falconer *et al.*, 1994). Characteristic tissue injury resulted from experimental administration of the extract to mice, including liver, kidney and intestinal damage which was consistent with previous reports of cylindrospermopsin poisoning in humans.

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